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Citation for final published version:

Edwards, Sarah C., Sutton, Caroline E., Ladell, Kristin ORCID: <https://orcid.org/0000-0002-9856-2938>, Grant, Emma J., McLaren, James E. ORCID: <https://orcid.org/0000-0002-7021-5934>, Roche, Fiona, Dash, Pradyot, Apiwattanakul, Nopporn, Awad, Walid, Miners, Kelly L., Lalor, Stephen J., Ribot, Julie C., Baik, Song, Moran, Barry, McGinley, Aoife, Pivorunas, Valerie, Dowding, Lori, Macoritto, Michael, Paez-Cortez, Jesus, Slavin, Anthony, Anderson, Graham, Silva-Santos, Bruno, Hokamp, Karsten, Price, David A. ORCID: <https://orcid.org/0000-0001-9416-2737>, Thomas, Paul G., McLoughlin, Rachel M. and Mills, Kingston H.G. 2020. A population of proinflammatory T cells coexpresses α and γ T cell receptors in mice and humans. *Journal of Experimental Medicine* 217 (5) , e20190834. 10.1084/jem.20190834 file

Publishers page: <http://dx.doi.org/10.1084/jem.20190834>
<<http://dx.doi.org/10.1084/jem.20190834>>

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BRIEF DEFINITIVE REPORT

A population of proinflammatory T cells coexpresses $\alpha\beta$ and $\gamma\delta$ T cell receptors in mice and humans

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T cells are classically recognized as distinct subsets that express $\alpha\beta$ or $\gamma\delta$ TCRs. We identify a novel population of T cells that coexpress $\alpha\beta$ and $\gamma\delta$ TCRs in mice and humans. These hybrid $\alpha\beta$ - $\gamma\delta$ T cells arose in the murine fetal thymus by day 16 of ontogeny, underwent $\alpha\beta$ TCR-mediated positive selection into CD4⁺ or CD8⁺ thymocytes, and constituted up to 10% of TCR δ ⁺ cells in lymphoid organs. They expressed high levels of IL-1R1 and IL-23R and secreted IFN- γ , IL-17, and GM-CSF in response to canonically restricted peptide antigens or stimulation with IL-1 β and IL-23. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells were transcriptomically distinct from conventional $\gamma\delta$ T cells and displayed a hyperinflammatory phenotype enriched for chemokine receptors and homing molecules that facilitate migration to sites of inflammation. These proinflammatory T cells promoted bacterial clearance after infection with *Staphylococcus aureus* and, by licensing encephalitogenic Th17 cells, played a key role in the development of autoimmune disease in the central nervous system.

Introduction

MHC-restricted CD4⁺ and CD8⁺ T cells typically mediate pathogen-specific adaptive immunity and express $\alpha\beta$ TCRs. In contrast, $\gamma\delta$ T cells play an important role in innate immunity at mucosal surfaces but can also display features of immunological memory, analogous to conventional $\alpha\beta$ T cells (Misiak et al., 2017; Sutton et al., 2009). The accepted dogma is that common lymphoid progenitors develop into cells that express either $\alpha\beta$ or $\gamma\delta$ TCRs and that each population subsequently occupies a specific and highly conserved niche within the immune system.

$\gamma\delta$ T cells are required for optimal innate and adaptive immune responses to infection and tumors (Murphy et al., 2014; Rei et al., 2014; Silva-Santos et al., 2015). They are the first lymphocytes to emerge in the fetus, and before full maturation of the immune system, they mediate protective functions in young animals (Shibata et al., 2007; Sinkora et al., 2005). A unique feature of murine $\gamma\delta$ T cells is the preferential expression of different TCR γ variable region (V γ) segments in different tissues. For example, V γ 5⁺ $\gamma\delta$ T cells are present in skin, V γ 6⁺ $\gamma\delta$

T cells localize to the reproductive mucosa, and V γ 1⁺ or V γ 4⁺ $\gamma\delta$ T cells are found in secondary lymphoid organs (nomenclature of Heilig and Tonegawa, 1986). $\gamma\delta$ T cells produce an array of cytokines, including IFN- γ , IL-4, IL-17A, IL-17F, IL-21, IL-22, GM-CSF, and TNF- α (Lockhart et al., 2006; Ribot et al., 2009; Sutton et al., 2012).

Although $\gamma\delta$ T cells display characteristics of adaptive memory, they can also produce IL-17 upon stimulation with IL-1 β and IL-23 in the absence of TCR engagement and provide an early source of innate proinflammatory cytokines that help amplify T helper type 17 (Th17) responses in certain autoimmune and infectious diseases (Conti et al., 2014; Crowley et al., 1997; Sutton et al., 2009). In humans with multiple sclerosis, increased frequencies of $\gamma\delta$ T cells have been detected in acute brain lesions (Hvas et al., 1993; Wucherpfennig et al., 1992), and clonal expansions of $\gamma\delta$ T cells have been observed in cerebrospinal fluid during the early stages of disease (Shimonkevitz et al., 1993). Similarly, IL-17-producing V γ 4⁺ T cells infiltrate

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the brain and spinal cord of mice with experimental autoimmune encephalomyelitis (EAE; [Price et al., 2012](#); [Sutton et al., 2009](#)). $V\gamma 4^+$ T cells also mediate inflammation via IL-17 production in the dermis of mice with psoriasis ([Cai et al., 2011](#)) and accumulate in the draining LNs and joints of mice with collagen-induced arthritis ([Roark et al., 2007](#)).

In this study, we identified a discrete population of T cells that coexpressed $\alpha\beta$ and $\gamma\delta$ TCRs. These hybrid $\alpha\beta$ - $\gamma\delta$ T cells were transcriptomically distinct from conventional $\gamma\delta$ T cells, poised to migrate to sites of inflammation, and responsive to MHC class I (MHCI)-restricted or MHCII-restricted peptide antigens or stimulation with IL-1 β and IL-23. In line with these findings, hybrid $\alpha\beta$ - $\gamma\delta$ T cells protected against infection with *Staphylococcus aureus* and, by licensing encephalitogenic Th17 cells, triggered autoimmune pathology in the central nervous system (CNS).

Results and discussion

Identification of hybrid $\alpha\beta$ - $\gamma\delta$ T cells

Initial flow cytometric analyses with antibodies specific for the constant regions of TCR δ and TCR β unexpectedly revealed a rare population of TCR δ^+ TCR β^+ cells in the LNs of WT C57BL/6 mice ([Fig. 1 A](#) and [Fig. S1 A](#)). These findings were substantiated using confocal microscopy, which demonstrated surface expression of TCR β on purified TCR δ^+ cells ([Fig. S1 B](#)), and RT-PCR, which demonstrated the presence of transcripts encoding the joining region of TCR β (*Trb*) in purified TCR δ^+ cells ([Fig. S1 C](#)). Moreover, TCR δ^+ cells coexpressed TCR β and various TCR α variable region (*V α*) segments ([Fig. 1 B](#)), and some $V\gamma 4^+$, $V\gamma 1^+$, and $V\gamma 4$ - $V\gamma 1^-$ T cells coexpressed TCR δ and TCR β ([Fig. 1 C](#) and [Fig. S1 D and E](#)). ImageStream experiments confirmed coexpression of $V\gamma 4$ with TCR δ and TCR β at the single-cell level ([Fig. 1 D](#)). In addition, flow cytometric analyses revealed that ~6% of $V\gamma 4^+$ TCR δ^+ cells expressed TCR β ([Fig. S1 F](#)). These hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed higher levels of CD3 ϵ than TCR δ^+ TCR β^- cells ([Fig. 1 E](#)), together with either CD4 or CD8 α ([Fig. 1 F](#)). Coexpression of $\alpha\beta$ and $\gamma\delta$ TCRs was further validated using TCR $\alpha^{-/-}$ and TCR $\beta^{-/-}$ mice, which lacked hybrid $\alpha\beta$ - $\gamma\delta$ T cell populations, whereas ~7% of $V\gamma 4^+$ TCR δ^+ cells in WT mice expressed TCR β ([Fig. 1 G](#)). Importantly, $V\gamma 4^+$ TCR δ^+ TCR β^+ cells were present in MHCI $^{-/-}$ and MHCII $^{-/-}$ mice ([Fig. 1 G](#)). CD4 $^+$ $V\gamma 4^+$ TCR δ^+ TCR β^+ cells were absent in MHCII $^{-/-}$ mice, whereas CD8 $^+$ $V\gamma 4^+$ TCR δ^+ TCR β^+ cells were absent in MHCI $^{-/-}$ mice ([Fig. S1 G](#)). Double-negative cells were the most predominant population in each strain ([Fig. S1 G](#)). An examination of the ontogeny of hybrid $\alpha\beta$ - $\gamma\delta$ T cells revealed that $V\gamma 4^+$ cells emerged in the thymus between embryonic days (E) 14 and 16 (E14 and E16; [Fig. 1 H](#)). Although the majority of these $V\gamma 4^+$ cells were TCR δ^+ TCR β^- (conventional $\gamma\delta$ cells), we detected a clear population of $V\gamma 4^+$ cells that coexpressed TCR β . These findings suggest that hybrid $\alpha\beta$ - $\gamma\delta$ T cells develop in the embryonic thymus in synchrony with the early waves of conventional $\gamma\delta$ T cells, following the same CD4 and CD8 expression patterns as $\gamma\delta$ (double negative) or $\alpha\beta$ T cells (MHC restricted).

Flow cytometric analyses of human peripheral blood mononuclear cells (PBMCs) revealed a rare population of cells (<1% of

CD3 $^+$ T cells) that coexpressed TCR $\alpha\beta$ and V $\delta 2$ ([Fig. 1 I](#)) and further showed that ~10% of all circulating V $\delta 2^+$ cells expressed TCR $\alpha\beta$ ([Fig. 1 J](#)). These data provide preliminary evidence that hybrid $\alpha\beta$ - $\gamma\delta$ T cells are also present in humans.

Molecular analysis of TCR expression in $V\gamma 4^+$ TCR β^+ cells

To examine the molecular basis of $\alpha\beta$ and $\gamma\delta$ TCR coexpression, we used an unbiased approach ([Quigley et al., 2011](#)) to amplify all expressed *Tra*, *Trb*, *Trg*, and *Trd* gene rearrangements in flow-purified $V\gamma 4^+$ TCR β^+ cells. Analysis of the *Tra* and *Trb* gene expression profiles revealed substantial heterogeneity ([Fig. 2 A](#)). We also detected *Trgv2* gene transcripts, presumably reflecting antibody cross-recognition of V $\gamma 2$ and V $\gamma 4$. The associated *Trd* gene transcripts were heavily biased toward *Trdv2-2* and *Trdv5*. Of note, disruption of a single chromatin loop facilitates “default” rearrangements with *Trdv2-2* ([Chen et al., 2015](#)), which also occur in ~42% of thymocytes in WT mice, and *Trdv5* is located in a reversed transcriptional orientation 3' of the *Trdc* gene ([Glusman et al., 2001](#)).

To confirm these findings, we flow-sorted single $V\gamma 4^+$ TCR δ^+ TCR β^+ cells from the LN cells of mice with EAE ([Fig. 2 B](#)). Functional *Tra*, *Trb*, *Trg*, and *Trd* gene rearrangements, consistent with the index sort parameters, were detected in ~9% (7/79) of these cells ([Fig. 2 C](#)). In line with the population-level data, *Trgv2* rearranged exclusively with *Trgj2*, *Trgv4* rearranged exclusively with *Trgj1*, and *Trdv2-2* and *Trdv5* rearranged exclusively with *Trdj1*. Moreover, the CDR3 γ and CDR3 δ loop sequences were highly conserved, irrespective of gene use, and matched those identified in a recent study of CCR6 $^+$ CD27 $^-$ $\gamma\delta$ T cells ([Kashani et al., 2015](#)). No clear patterns were observed among *Tra* and *Trb* gene transcripts amplified from single $V\gamma 4^+$ TCR δ^+ TCR β^+ cells. These data show that hybrid $\alpha\beta$ - $\gamma\delta$ T cells coexpress diverse TCR α and TCR β chains and a limited array of TCR γ and TCR δ chains.

Hybrid $\alpha\beta$ - $\gamma\delta$ T cells can be activated innately or via $\alpha\beta$ or $\gamma\delta$ TCRs

We then addressed the functionality of hybrid $\alpha\beta$ - $\gamma\delta$ T cells after stimulation with IL-1 β and IL-23, which are known to promote the activation of innate $\gamma\delta$ T cells ([Sutton et al., 2009](#)), or ligation of the expressed $\alpha\beta$ or $\gamma\delta$ TCRs. $V\gamma 4^+$ T cells produced IFN- γ and IL-17 in response to IL-1 β and IL-23 ([Fig. 3 A](#)). Moreover, depletion of $V\gamma 4^+$ cells from the CD3 $^+$ T cell pool abrogated IFN- γ and IL-17 production elicited by IL-1 β and IL-23 ([Fig. 3 A](#)), and $V\gamma 4^+$ hybrid $\alpha\beta$ - $\gamma\delta$ T cells more frequently expressed IL-1R1 or IL-23R ([Fig. 3 B](#)) or coexpressed IL-1R1 and IL-23 ([Fig. S2 A](#)) compared with conventional $V\gamma 4^+$ $\gamma\delta$ or $\alpha\beta$ T cells. Stimulation of hybrid $\alpha\beta$ - $\gamma\delta$ T cells with IL-1 β and IL-23 induced the expression of *Ifng* and *Il17a* ([Fig. 3 C](#)), as well as the production of IFN- γ and IL-17 ([Fig. S2 B](#)). Although *Ifng* and *Il17a* expression was detected in the absence of TCR signaling, coactivation with anti-CD3 enhanced IFN- γ expression at the gene and protein levels. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells also expressed *Rorc*, which encodes ROR γ t, the transcription factor associated with IL-17 production ([Ivanov et al., 2006](#)), and *Sox13*, which encodes the transcription factor associated with IL-17-producing $V\gamma 4^+$ $\gamma\delta$ T cells ([Gray et al., 2013](#)), after stimulation with IL-1 β and IL-23 ([Fig. S2 C](#)).

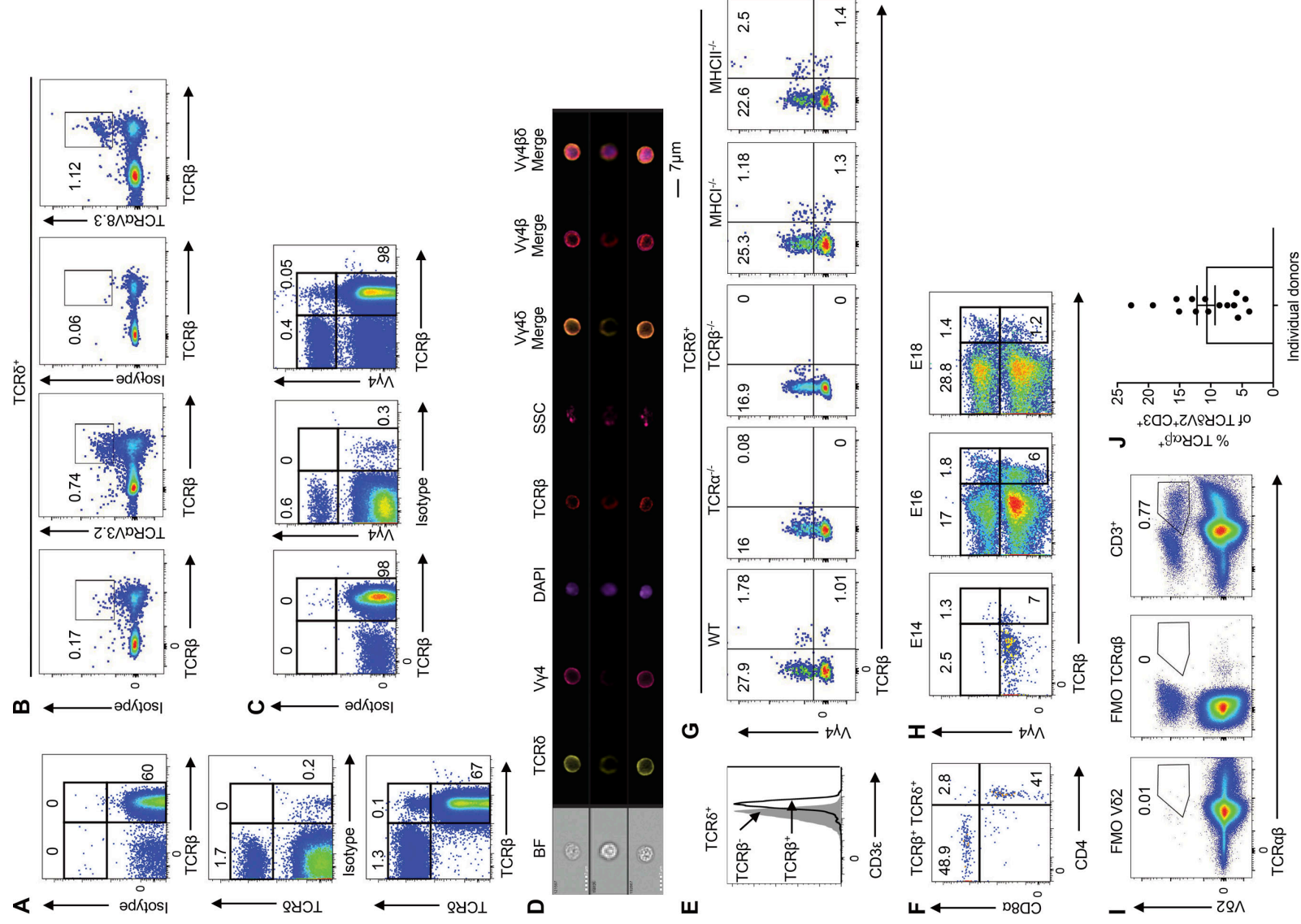


Figure 1. Identification of unconventional T cells that coexpress $\alpha\beta$ and $\gamma\delta$ TCRs. (A and B) Flow cytometric analysis of LN cells from WT mice, stained for TCR δ and TCR β , gated on live CD3 $^{+}$ T cells (A), or stained for V α 2, V α 8.3, and TCR β , gated on live CD3 $^{+}$ TCR δ^{+} cells (B). (C) Flow cytometric analysis of LN cells from WT mice, stained for V γ 4 and TCR β , gated on live CD3 $^{+}$ T cells. (D) ImageStream analysis of LN cells from WT mice, stained for V γ 4, TCR δ , and TCR β , and costained with DAPI. (E and F) Flow cytometric analysis of LN cells from WT mice, showing mean fluorescence intensity (MFI) for CD3 ϵ expression, gated on live CD3 $^{+}$ TCR δ^{+} TCR β^{-} or CD3 $^{+}$ TCR δ^{+} TCR β^{+} cells (E), or stained for CD4 and CD8 α , gated on live CD3 $^{+}$ TCR δ^{+} TCR β^{+} cells (F). (G) Flow cytometric analysis of LN cells from WT, TCR $\alpha^{-/-}$, TCR $\beta^{-/-}$, MHCII $^{-/-}$, or MHCII $^{-/-}$ mice, stained for V γ 4 and TCR β , gated on live CD3 $^{+}$ TCR δ^{+} cells. (H) Flow cytometric analysis of thymocytes isolated from WT mice on E14, E16, and E18, stained for V γ 4 and TCR β , gated on live CD3 $^{+}$ cells. The gating strategy (A–H) is shown in Fig. S1 A. (I) Flow cytometric analysis of human PBMCs showing expression of TCR $\alpha\beta$ and V δ 2, gated on live CD3 $^{+}$ cells. (J) Percent expression of TCR $\alpha\beta$ among V δ 2 $^{+}$ cells in human PBMCs ($n = 15$ healthy donors), gated on live CD3 $^{+}$ cells. Data are representative of two independent experiments. Flow cytometry plots are representative of at least three independent experiments ($n = 18$ samples). BF, brightfield; FMO, fluorescence minus one; SSC, side scatter.

Next, we examined the ability of hybrid $\alpha\beta$ - $\gamma\delta$ T cells to respond to MHCII-restricted antigens, initially using OT-II transgenic mice, which exclusively express $\alpha\beta$ TCRs that recognize an OVA peptide restricted by I-A b . Purified TCR δ^{+} cells from OT-II mice, but not WT mice, produced IFN- γ in response to dendritic cells (DCs) pulsed with OVA, but not with KLH (Fig. S2 D). Moreover, purified TCR δ^{+} TCR β^{+} cells, but not TCR δ^{+} TCR β^{-} cells, from OT-II mice produced IFN- γ , IL-17, and GM-CSF (Fig. 3 D) and significantly up-regulated *Tbx21*, *Rorc*, *Stat3*, and *Mcam* in response to OVA (Fig. S2 E). Co-stimulation of TCR δ^{+} TCR β^{+} cells from OT-II mice with IL-1 β and IL-23 in the presence of OVA-pulsed DCs enhanced the production of IFN- γ and GM-CSF, which was reversed by the addition of anti-MHCII (Fig. 3 E). OVA stimulation reduced the production of IL-17 induced by IL-1 β and IL-23 (Fig. 3 E), potentially reflecting enhanced expression of *Tbx21*, which encodes T-bet, a transcription factor that blocks Runx1-mediated transactivation of the *Rorc* promoter and subsequent expression of IL-17. Stimulation of OT-II-derived $\gamma\delta$ T cells with OVA under Th1 and Th2 polarizing conditions also enhanced the production of IFN- γ and IL-4 (Fig. S2 F). Further evidence of MHCII-restricted peptide recognition by hybrid $\alpha\beta$ - $\gamma\delta$ T cells was obtained using V γ 4 $^{+}$ TCR β^{+} cells purified from WT mice immunized with myelin oligodendrocyte protein (MOG) and CFA. IL-1 β and IL-23 induced the expression of *Tbx21* and *Rorc* (Fig. 3 F), as well as the production of IFN- γ , IL-17, and GM-CSF, which was substantially enhanced in the presence of MOG and DCs (Fig. 3, F and G; and Fig. S2 G).

The antigen specificity of hybrid $\alpha\beta$ - $\gamma\delta$ T cells was confirmed using the corresponding MOG tetramer to stain LN cells 7 d after the induction of EAE. Approximately 33% of V γ 4 $^{+}$ TCR β^{+} cells stained with the MOG tetramer, in contrast to ~12% of conventional $\alpha\beta$ T cells (Fig. S2 H). Moreover, we found that purified $\gamma\delta$ T cells from OT-I mice, which harbor transgenic CD8 $^{+}$ T cells specific for OVA_{257–264} presented by H-2K b , produced IFN- γ in the presence of OVA-pulsed DCs and IL-12 (Fig. S2 I). These data suggest that hybrid $\alpha\beta$ - $\gamma\delta$ T cells respond in an innate-like manner to IL-1 β and IL-23, akin to conventional $\gamma\delta$ T cells, but also recognize peptide antigens presented by MHCII or MHCI, akin to conventional $\alpha\beta$ T cells.

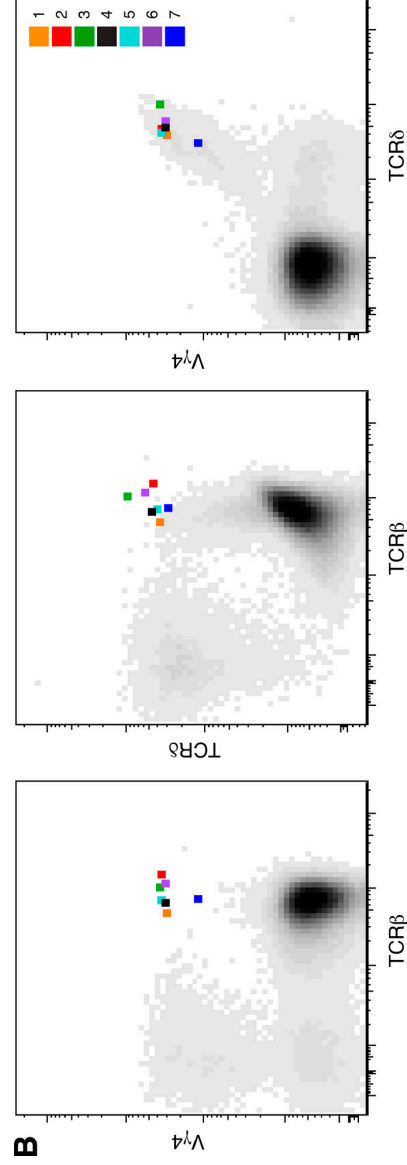
The lack of well-defined antigens recognized by $\gamma\delta$ T cells precluded analysis of activation via $\gamma\delta$ TCRs. Nonetheless, we examined the expression of the natural killer group 2D (NKG2D) receptor, which is crucial for innate-like lymphocyte recognition of stressed or infected cells (Correia et al., 2013). Hybrid V γ 4 $^{+}$ $\alpha\beta$ - $\gamma\delta$ T cells expressed higher levels of NKG2D than conventional V γ 4 $^{+}$ $\gamma\delta$ or $\alpha\beta$ T cells (Fig. S2 J). Expression of Ki67,

a marker of proliferation, was also significantly enhanced in hybrid V γ 4 $^{+}$ $\alpha\beta$ - $\gamma\delta$ T cells, but not in conventional V γ 4 $^{+}$ $\gamma\delta$ T cells, after co-culture with YAC-1 cells, which constitutively express NKG2D ligands (Fig. S2 K). Moreover, stimulation of TCR δ^{+} TCR β^{+} cells with an activating anti-TCR δ antibody significantly increased the expression of *Ifng*, *Il17a*, *Csf2*, *Mcam*, *Tbx21*, *Rorc*, and *Il1r1* (Fig. S2 L), as well as the production of IL-17 driven by IL-1 β and IL-23 (Fig. S2 M).

Further phenotypic analyses revealed that hybrid $\alpha\beta$ - $\gamma\delta$ T cells displayed unique properties and certain features associated with conventional $\gamma\delta$ T cells. The majority of hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed CD27 (Fig. S3 A), whereas V γ 4 $^{+}$ $\gamma\delta$ T cells were either CD27 $^{+}$ or CD27 $^{-}$, demarcating the production of IFN- γ or IL-17, respectively (Ribot et al., 2009). Moreover, CCR6, a chemokine receptor expressed on IL-17-secreting $\gamma\delta$ T cells (Haas et al., 2009; Papotto et al., 2017) and pathogenic Th17 cells during EAE (Reboldi et al., 2009), was expressed by hybrid $\alpha\beta$ - $\gamma\delta$ and conventional V γ 4 $^{+}$ $\gamma\delta$ T cells, but not by $\alpha\beta$ T cells (Fig. S3 A). Similarly, CCR2, which allows Th17 cells to cross the blood–brain barrier and is associated with the development of EAE (Kara et al., 2015), was more commonly expressed by hybrid $\alpha\beta$ - $\gamma\delta$ T cells relative to conventional $\gamma\delta$ or $\alpha\beta$ T cells. The integrin CD49d, a subunit of the cell adhesion molecule α 4 β 1, very late antigen-4 (VLA-4), which is involved in the migration of encephalitogenic T cells into the CNS (Yednock et al., 1992), was expressed by ~40% of hybrid $\alpha\beta$ - $\gamma\delta$ T cells but also by ~20% of conventional V γ 4 $^{+}$ $\gamma\delta$ T cells (Fig. S3 A). In addition, hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed IL-2R, CD25, and CD122 at higher frequencies than conventional V γ 4 $^{+}$ $\gamma\delta$ T cells or $\alpha\beta$ T cells (Fig. S3 A). These observations indicate that hybrid $\alpha\beta$ - $\gamma\delta$ T cells share some phenotypic features with $\gamma\delta$ T cells but also exhibit characteristics typically associated with cell activation and migration.

Hybrid $\alpha\beta$ - $\gamma\delta$ T cells protect against *S. aureus* infection

To probe the biological relevance of these findings, we examined the ability of hybrid $\alpha\beta$ - $\gamma\delta$ T cells to protect against infection with *S. aureus* in a murine model, where a key role has been defined for IL-17-producing $\gamma\delta$ T cells (Murphy et al., 2014). Mice infected with *S. aureus* displayed significantly elevated concentrations of IL-17 and IL-1 β in the peritoneal fluid 3 h after infection (Fig. 3 H). At the same time point, there was a non-significant increase in the absolute number of TCR δ^{+} cells (Fig. 3 I) and a significant increase in the absolute number of V γ 4 $^{+}$ TCR β^{+} cells in the peritoneal cavity (Fig. 3 J). In addition, there was a significant increase in IFN- γ -producing and a nonsignificant increase in IL-17-producing V γ 4 $^{+}$ TCR δ^{+} TCR β^{+} cells



Cell	Trav	CDR3 α	Traj	TbV	CDR3 β	TrbJ	TrgV	CDR3 γ	TrgJ	TrdV	CDR3 δ	TrdJ
1	9D-3/9N-4	AVTGNYYK	40	5	SQDQTEV	1-1	4	GYSSGFHK	1	5	GDIGGIRATDKL	1
2	6-6/6D-6/6D-7	SAPNSNRI	31	13-3	SDRDQDTQ	2-5	4	GLYSSGFHK	1	5	GVIGGIRATDKL	1
3	14D-3/DV8/14N-3	SRNAYKV	30	13-2	GGPTGQDPTQ	2-5	4	GLYSSGFHK	1	2-2	MERAGGIRATDKL	1
4	5D-4	LWNSNRI	31	3	SEGLGVYEQ	2-7	2	WYSSGFHK	2	5	GDIGGIRATDKL	1
5	6D-6/6N-6	SDRPXNYVL	21	29	SGTNVL	2-4	4	GLYSSGFHK	1	5	GLIGGIRATDKL	1
6	14D-3/DV8	SDAGSNAKL	42	2	SQDLAETL	2-3	2	WANS SGFKH	2	2-2	MERDIGGIRATDKL	1
7	3-4	RTGNYYK	40	26	SLGLGYEQ	2-7	2	WHSSGFHK	2	4/DV7	PYRRDSTDKL	1
			2	2	SOEGYAEQ	2-1	4	GORGYS GFHK	1			

Figure 2. Molecular analysis of TCR expression in flow-purified V γ 4⁺TCR β ⁺ cells. Viable CD3⁺V γ 4⁺TCR β ⁺ cells were flow purified from WT mice. **(A)** Pie charts display relative population-level frequencies for the *Trav*, *Trbv*, *Trgv*, and *Trdv* genes depicted in the key (IMGT nomenclature), with the total number of sequences indicated below. Concatenated data are shown ($n = 4$ mice). **(B)** Flow cytometry plots showing index-sorted V γ 4⁺TCR δ ⁺TCR β ⁺ cells superimposed on cloud plots depicting the overall distribution of V γ 4 versus TCR β (left panel), TCR δ versus TCR β (middle panel), and V γ 4 versus TCR δ (right panel) in one mouse with EAE. **(C)** TCR sequences amplified from the single cells shown in B. Cells and sequences are color matched in B and C.

in infected versus naive mice (Fig. 3 K). Adoptive transfer of ~10,000 flow-purified V γ 4⁺TCR β ⁺ cells from WT mice to IL-17A^{-/-} mice before infection with *S. aureus* significantly reduced bacterial load in the peritoneal cavity and systemic dissemination to the kidney (Fig. 3 L). These data demonstrate that hybrid $\alpha\beta$ - $\gamma\delta$ T cells play a protective role in immunity to *S. aureus* infection.

Hybrid $\alpha\beta$ - $\gamma\delta$ T cells play a critical role in the development of EAE

The role of $\gamma\delta$ T cells in autoimmune diseases is well established (Sutton et al., 2009). We found that hybrid $\alpha\beta$ - $\gamma\delta$ T cells were significantly expanded and secreted IL-17 in the LNs of WT mice 3–10 d after the induction of EAE (Fig. 4, A and B). Hybrid $\alpha\beta$ - $\gamma\delta$ T cells also dominated the V γ 4⁺ compartment in the CNS of naive mice, and the number of hybrid $\alpha\beta$ - $\gamma\delta$ T cells in the CNS was significantly increased 10 d after the induction of EAE (Fig. 4 C). Conventional $\gamma\delta$ T cells also infiltrated the brain 7–10 d after challenge, but CD4⁺ $\alpha\beta$ T cells were numerically dominant by day 10 (Fig. 4 C). Using a validated in vivo staining technique with an anti-CD45 antibody administered i.v. immediately before sacrifice, we demonstrated that hybrid $\alpha\beta$ - $\gamma\delta$ T cells were the dominant CNS tissue-resident T cells in naive mice (Fig. 4 D) and that TCR δ ⁺TCR β ⁺ cells outnumbered TCR δ ⁺TCR β ⁻ cells on day 10 of EAE (Fig. 4 E). These CNS-infiltrating hybrid $\alpha\beta$ - $\gamma\delta$ T cells displayed an activated, proliferative phenotype (Fig. 4 F) and secreted IL-17 (Fig. 4 G). Early in EAE (days 3–7), hybrid $\alpha\beta$ - $\gamma\delta$ T cells were the dominant IL-17-secreting subset in the CNS. However, conventional $\gamma\delta$ T cells and CD4⁺ T cells were also important sources of IL-17 in the CNS, especially as the disease developed over time, and CD4⁺ $\alpha\beta$ T cells formed the dominant IL-17-secreting population on day 10 of EAE (Fig. 4 G).

Depletion of V γ 4⁺ cells in vivo, which removes conventional V γ 4⁺ $\gamma\delta$ and hybrid V γ 4⁺ $\alpha\beta$ - $\gamma\delta$ T cells, significantly impaired the development of EAE (Fig. 4 H) and limited the infiltration of cytokine-producing CD4⁺ T cells into the CNS (Fig. 4 I). To address the specific contribution of V γ 4⁺ hybrid $\alpha\beta$ - $\gamma\delta$ T cells in this model, we depleted V γ 4⁺TCR β ⁺ cells from the spleens and LNs of mice immunized with MOG and CFA. Adoptive transfer of unmanipulated T cells from MOG-immunized mice after expansion in vitro with MOG, IL-1 β , and IL-23 induced EAE. Depletion of V γ 4⁺TCR β ⁺ cells significantly reduced the production of IFN- γ , IL-17, and GM-CSF in culture supernatants after incubation with MOG, IL-1 β , and IL-23 (Fig. 4 J) and abrogated the induction of EAE (Fig. 4 K). This attenuated disease course was associated with significantly reduced frequencies and absolute numbers of various cytokine-producing T cells in the CNS (Fig. 4, L and M). To address the contribution of hybrid V γ 4⁺ $\alpha\beta$ - $\gamma\delta$ T cells in the priming of encephalitogenic CD4⁺ T cells, we depleted hybrid V γ 4⁺ $\alpha\beta$ - $\gamma\delta$ T cells from a culture of LN and

spleen cells from MOG-immunized mice before or after culture with MOG, IL-1 β , and IL-23. Depletion of hybrid $\alpha\beta$ - $\gamma\delta$ T cells before, but not after, culture significantly impaired the development of EAE following adoptive transfer to naive mice (Fig. 4 N). Cells depleted of hybrid $\alpha\beta$ - $\gamma\delta$ T cells before culture with MOG, IL-1 β , and IL-23 also expressed significantly lower amounts of *Itga4*, which encodes the essential CD4⁺ T cell trafficking molecule VLA-4 (Fig. 4 O). Moreover, antigen-specific CD4⁺ T cells cultured with MOG, IL-1 β , and IL-23 in the presence of hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed significantly more IFN- γ (Fig. 4 P) and *Ifng* (Fig. 4 Q) and proliferated to a greater extent (Fig. 4 R) than CD4⁺ T cells cultured in the absence of hybrid $\alpha\beta$ - $\gamma\delta$ T cells. These findings demonstrate that hybrid V γ 4⁺ $\alpha\beta$ - $\gamma\delta$ T cells play a nonredundant role in the immunopathogenesis of EAE via their ability to migrate to inflammatory sites and prime encephalitogenic CD4⁺ T cells.

Hybrid $\alpha\beta$ - $\gamma\delta$ T cells display a hyperactivated phenotype

Although hybrid $\alpha\beta$ - $\gamma\delta$ T cells are outnumbered by conventional $\gamma\delta$ T cells, they are rapidly mobilized and play a key pathogenic role in development of CNS inflammation in the EAE model. To understand these proinflammatory effects in more detail, we performed a transcriptomic analysis of hybrid $\alpha\beta$ - $\gamma\delta$ T cells and conventional $\gamma\delta$ T cells isolated from the LNs of WT mice under physiological conditions and during EAE.

The data revealed that 1,259 genes were differentially expressed in hybrid $\alpha\beta$ - $\gamma\delta$ T cells (V γ 4⁺TCR β ⁺) relative to conventional $\gamma\delta$ T cells (V γ 4⁺TCR β ⁻) under physiological conditions and that the majority ($n = 1,184$) of these differentially expressed genes (DEGs) were up-regulated in hybrid $\alpha\beta$ - $\gamma\delta$ T cells relative to conventional $\gamma\delta$ T cells (Fig. 5, A and B). Functional enrichment analysis revealed that transcripts associated with T cell activation, cellular migration, cytokine stimulation, and immune responses to foreign stimuli were enriched in hybrid $\alpha\beta$ - $\gamma\delta$ T cells relative to conventional $\gamma\delta$ T cells (Fig. 5 C and Fig. S3 B). Genes associated with the $\alpha\beta$ T cell phenotype, including *Cd4*, *Cd8*, *Cd6*, and *Cd28*, were expressed at higher levels in hybrid $\alpha\beta$ - $\gamma\delta$ T cells relative to conventional $\gamma\delta$ T cells. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells also expressed higher levels of genes associated with cellular trafficking, including *Ccr7*, *Cx3cr1*, *Ccl22*, and *Ccl17* (Fig. 5 C). In addition, hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed higher levels of *Myd88*, *Ly96*, *Thr2*, and *Thr13*, suggesting a capacity to respond to pathogen-associated molecular patterns, as well as genes associated with IL-1 signaling, including *Casp3*, *Il1b*, and *Il18* (Fig. 5 C). A selection of identified DEGs were validated by flow cytometry (Fig. 5 D).

Separation of hybrid $\alpha\beta$ - $\gamma\delta$ T cells from conventional $\gamma\delta$ T cells in the principal component analysis plot indicated differences in the corresponding gene expression profiles under physiological conditions and during EAE (Fig. 5 E). Differential

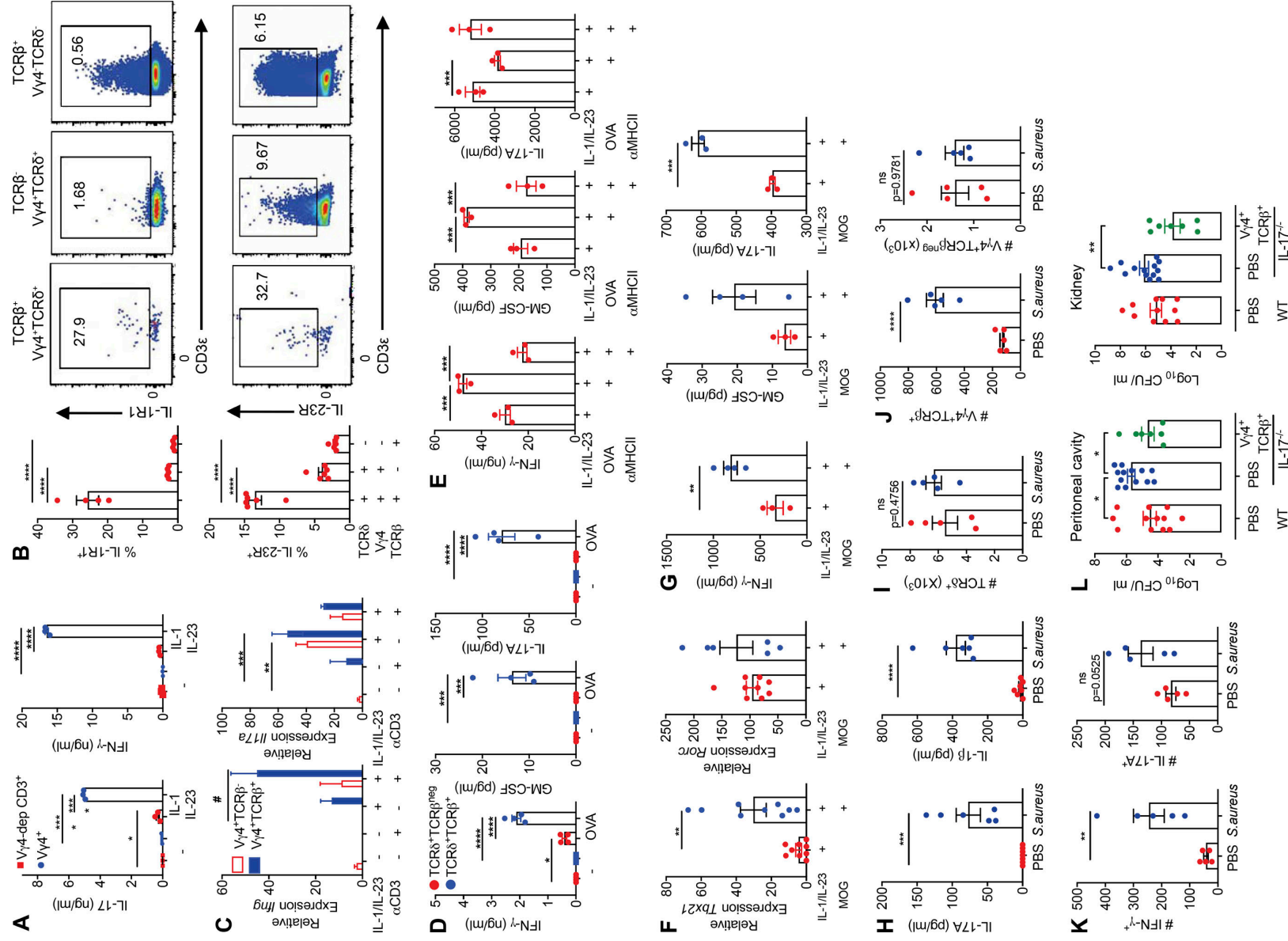


Figure 3. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells can be activated innately or via $\alpha\beta$ or $\gamma\delta$ TCRs. (A) Purified $V\gamma 4^+$ cells or $V\gamma 4$ -depleted $CD3^+$ cells were stimulated for 3 d with IL-1 β and IL-23 or with medium alone. Data show cytokine production measured by ELISA. (B) Flow cytometric analysis of IL-1R1 and IL-23R expression on $V\gamma 4^+TCR\delta^+TCR\beta^+$, $V\gamma 4^+TCR\delta^+TCR\beta^-$, or $V\gamma 4^+TCR\delta^-TCR\beta^+$ cells, gated on live $CD3^+$ cells. (C) Relative *Ifng* and *Il17a* expression in purified $V\gamma 4^+TCR\beta^+$ or $V\gamma 4^+TCR\beta^-$ cells stimulated for 3 d with IL-1 β , IL-2, and IL-23 in the presence or absence of plate-bound anti- $CD3$. (D and E) IFN- γ , IL-17, and GM-CSF production by $TCR\delta^+TCR\beta^+$ or $TCR\delta^+TCR\beta^-$ cells (D), or $TCR\delta^-TCR\beta^+$ cells (E), isolated from OT-II mice and stimulated for 3 d with or without OVA-pulsed DCs in the presence or absence of IL-1 β and IL-23 and/or anti-MHCII. Cytokines were measured by ELISA. (F and G) $V\gamma 4^+TCR\beta^+$ cells were isolated from MOG-immunized mice on day 7 and cultured for 3 d with DCs in the presence or absence of MOG and/or IL-1 β and IL-23. Relative gene expression was measured by RT-PCR (F), and cytokines were measured by ELISA (G). PECs were isolated from naive mice or 3 h after i.p. challenge with *S. aureus*. (H) Cytokine levels in PEC culture supernatants measured by ELISA. (I) Flow cytometric quantification of $TCR\delta^+$ cells, gated on live $CD3^+$ cells. (J) Flow cytometric quantification of $V\gamma 4^+TCR\delta^+TCR\beta^+$ or $V\gamma 4^+TCR\delta^+TCR\beta^-$ cells, gated on live $CD3^+TCR\delta^+$ cells. (K) Flow cytometric quantification of IFN- γ and IL-17 production by $V\gamma 4^+TCR\delta^+TCR\beta^+$ cells. (L) Bacterial loads in the peritoneal cavity and kidneys of WT mice 3 d after *S. aureus* infection or IL-17 $^{-/-}$ mice 3 d after adoptive transfer of $V\gamma 4^+TCR\beta^+$ cells from WT mice or mock transfer (PBS). Data are representative of three independent experiments ($n = 4$ – 6 in A–K) or combined from two experiments ($n = 8$ or 12 in L). Flow cytometry plots in B are representative of six samples. Results are shown as mean \pm SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; #, $P < 0.05$; ns, not significant; two-way ANOVA with Tukey's test for multiple comparisons (A, D, and E), one-way ANOVA with Tukey's test for multiple comparisons (B and L), three-way ANOVA with Tukey's test for multiple comparisons (C), or unpaired t test (F–K).

expression analysis was used to identify changes in gene expression levels for each cell type under each condition. DEGs were more common in hybrid $V\gamma 4^+$ $\alpha\beta$ - $\gamma\delta$ T cells (total, $n = 2,470$; up-regulated, $n = 157$; down-regulated, $n = 2,313$) compared with conventional $\gamma\delta$ T cells (total, $n = 652$; up-regulated, $n = 3$; down-regulated, $n = 649$), and most of these genes were similarly down-regulated in both cell types during EAE (Fig. S3 C). Enrichment analysis further revealed that these down-regulated genes were associated with T cell activation (cluster 2), cell cycle processes (cluster 3), and mRNA processing and transport (cluster 4; Fig. S3 C). In contrast, genes associated with cell migration and chemotaxis, including *Vcam1*, *Ccl2*, *Ccl8*, and *Cxcl13*, were preferentially up-regulated in hybrid $V\gamma 4^+$ $\alpha\beta$ - $\gamma\delta$ T cells from mice with EAE (Fig. 5, F and G; and Fig. S3, C and D). These data suggest that hybrid $\alpha\beta$ - $\gamma\delta$ T cells are transcriptomically distinct from conventional $\gamma\delta$ T cells, with a gene expression profile indicative of a proinflammatory and migratory phenotype.

Concluding remarks

Conventional $\alpha\beta$ and $\gamma\delta$ T cells originate from common thymocyte precursors, but the mechanisms that govern subsequent divergence and lineage fate are incompletely defined. The general consensus posits that a strong $\gamma\delta$ TCR-mediated signal directs thymocytes to the $\gamma\delta$ lineage, whereas a nonproductive signal permits TCR α rearrangements and commitment to the $\alpha\beta$ lineage (Hayes et al., 2005). However, aberrant expression of TCR chains has been demonstrated among $\alpha\beta$ and $\gamma\delta$ T cell populations (Bowen et al., 2014; Hochstenbach and Brenner, 1989; Ishida et al., 1990). For example, in-frame TCR δ rearrangements have been identified in $\alpha\beta$ T cells (Livak et al., 1995), and functional TCR β rearrangements have been detected in $\gamma\delta$ T cells (Bosco et al., 2008). The latter may even confer a proliferative advantage and selectively amplify certain subsets of murine TCR β^+ $\gamma\delta$ thymocytes (Wilson and MacDonald, 1998). Similarly, up to 50% of all V $\delta 1^+$ cells in humans are natural killer T (NKT)-like cells that express TCR α and TCR δ segments with TCR β (Pellicci et al., 2014). Of note, given the structure of the murine TCR α/δ locus, coexpression of $\alpha\beta$ and $\gamma\delta$ TCRs implies productive rearrangement of TCR α and TCR δ on different alleles. In addition, the murine TCR γ locus has been repeatedly

duplicated on chromosome 13, potentially facilitating multiple rearrangements (Glusman et al., 2001). It is also notable that dual-lineage lymphocytes expressing a B cell receptor and an $\alpha\beta$ TCR have recently been identified in humans and linked to the development of type 1 diabetes (Ahmed et al., 2019).

The collective data presented here identify a novel subset of hyperinflammatory T cells defined by the coexpression $\alpha\beta$ and $\gamma\delta$ TCRs. These intrathymically generated hybrid $\alpha\beta$ - $\gamma\delta$ T cells recognized MHC-restricted peptide antigens, like conventional $\alpha\beta$ T cells, and produced IFN- γ , IL-17, and GM-CSF in response to IL-1 β and IL-23, like conventional $\gamma\delta$ T cells, defining a niche at the interface between adaptive and innate immunity. Moreover, hybrid $\alpha\beta$ - $\gamma\delta$ T cells expressed chemokine receptors and homing molecules, facilitating migration to sites of inflammation. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells were expanded at the site of infection with *S. aureus*, and adoptive transfer of a very small number of purified hybrid $V\gamma 4^+$ $\alpha\beta$ - $\gamma\delta$ T cells from WT mice conferred protection against *S. aureus* infection in IL-17 $^{-/-}$ mice. In mice with EAE, IL-17-producing hybrid $\alpha\beta$ - $\gamma\delta$ T cells were found in the draining LNs before the appearance of IL-17-producing $\alpha\beta$ T cells. Depletion of hybrid $V\gamma 4^+$ $\alpha\beta$ - $\gamma\delta$ T cells suppressed the activation of Th17 cells and abrogated the development of EAE. These findings suggest that hybrid $\alpha\beta$ - $\gamma\delta$ T cells are equipped to act as highly proinflammatory “first responders,” illustrated here in the context of a bacterial infection and an autoimmune process in the CNS.

Materials and methods

Mice

C57BL/6 mice, IL-17 $^{-/-}$ (C57BL/6 background) mice, OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J), which exclusively express an $\alpha\beta$ TCR specific for OVA_{257–264} restricted by H-2K^b, and OT-II mice (C57BL/6-Tg(TcraTcrb)425Cbn/J), which exclusively express an $\alpha\beta$ TCR specific for OVA_{323–339} restricted by I-A^b, were sourced from the Jackson Laboratory. All mice were bred under specific pathogen-free conditions and maintained according to European Union Directives. Experiments were performed with sex-matched mice (aged 6–8 wk) under license B100/2412 from the Irish Health Protection Regulatory Agency with approval from the Trinity College Dublin Comparative Medicine Ethics

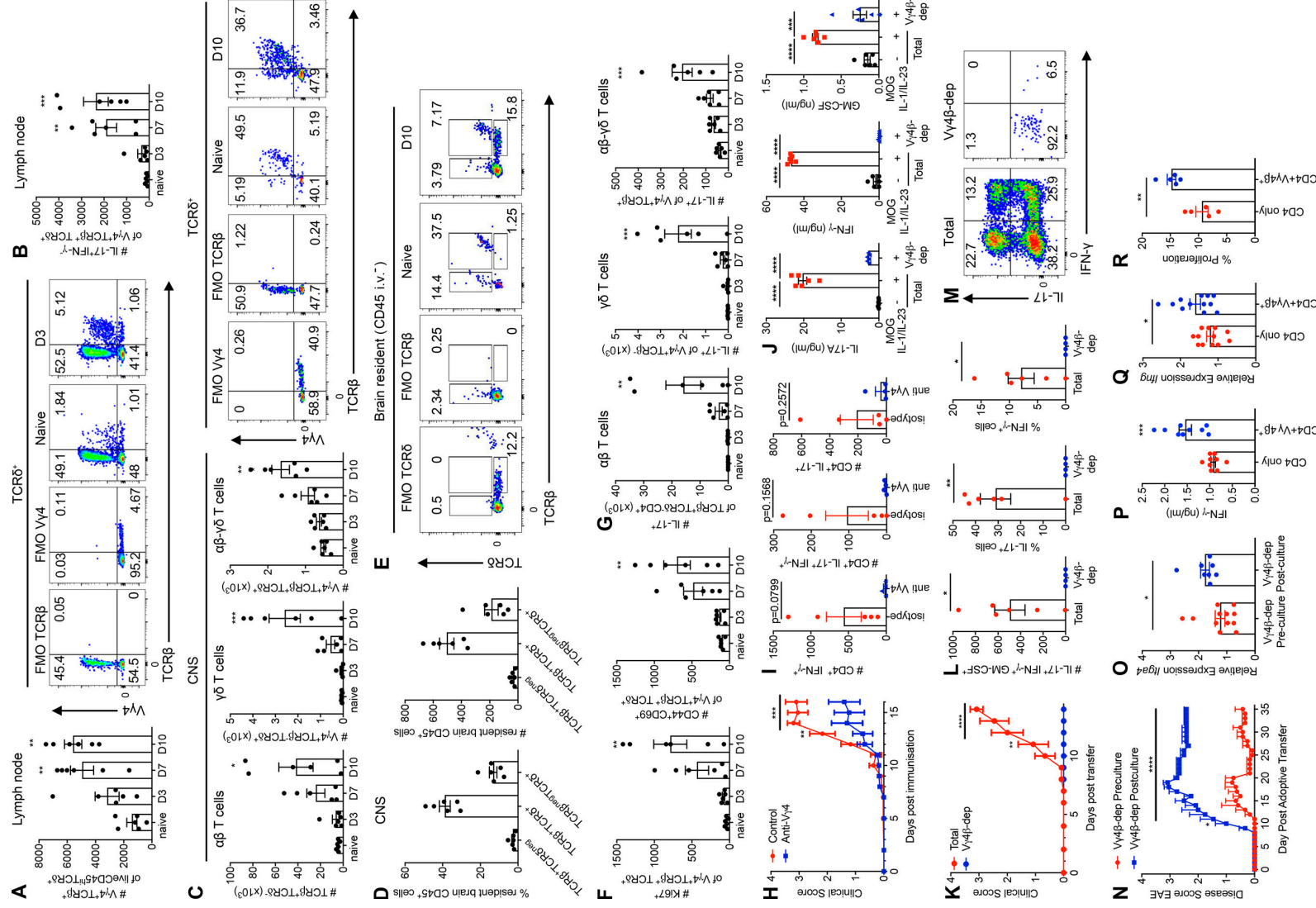


Figure 4. Hybrid V α 4⁺ $\alpha\beta$ - $\gamma\delta$ T cells promote CD4⁺ T cell homing to the CNS and drive the development of EAE. (A) Absolute numbers of V α 4⁺TCR δ ⁺TCR β ⁺ cells in the LNs of WT mice on days 0, 3, 7, and 10 of EAE. Right: representative flow cytometry plots. (B) Absolute numbers of IL-17-producing V α 4⁺TCR δ ⁺TCR β ⁺ cells in the LNs of WT mice on days 0, 3, 7, and 10 of EAE. (C) Absolute numbers of V α 4⁺TCR δ ⁺TCR β ⁺, V α 4⁺TCR δ ⁺TCR β ⁺, or V α 4⁺TCR δ ⁺TCR β ⁺ cells in the brains of WT mice on days 0, 3, 7, and 10 of EAE. Right: representative flow cytometry plots. (D) Absolute numbers and frequencies of brain-resident TCR δ ⁺TCR β ⁺, TCR δ ⁺TCR β ⁺, or TCR δ ⁺TCR β ⁺ cells after gating on CD45 i.v. cells from naive mice injected with anti-CD45 10 min before euthanasia. (E) Representative flow cytometry showing expression of TCR β versus TCR δ on tissue-resident (CD45 i.v.) T cells from naive mice or mice with EAE (day 10). (F) Absolute numbers of Ki67⁺ or CD44⁺CD69⁺ V α 4⁺TCR δ ⁺TCR β ⁺ cells in the CNS of WT mice on days 0, 3, 7, and 10 of EAE. (G) Absolute numbers of IL-17-producing V α 4⁺TCR δ ⁺TCR β ⁺, V α 4⁺TCR δ ⁺TCR β ⁺, or CD4⁺ V α 4⁺TCR δ ⁺TCR β ⁺ cells in the CNS of WT mice on days 0, 3, 7, and 10 of EAE. (H and I) WT mice were treated on days -1, 2, 5, 7, 11, and 14 with V α 4-depleting or isotype control antibodies. (H) Clinical scores for EAE. (I) Absolute numbers of cytokine-producing CD4⁺ T cells in the spinal cords on day 21 of EAE. (J–M) Total spleen and LN cells or V α 4⁺TCR β ⁺ flow-depleted (V α 4 β -dep) spleen and LN cells from MOG-immunized mice (day 7) were cultured for 3 d with MOG, IL-1 β , and IL-23. (J) IL-17, IFN- γ , and GM-CSF concentrations in culture supernatants quantified by ELISA. (K) Cultured T cells were transferred to naive mice and clinical scores were recorded for EAE. (L) Absolute numbers and frequencies of cytokine-producing cells in the brains of recipient mice on day 10 of EAE. (M) Representative flow cytometry plots showing production of IFN- γ versus IL-17, gated on live CD3⁺ cells. (N and O) V α 4⁺ TCR β ⁺ T cells were depleted from a culture of LN and spleen cells from MOG-immunized mice before or after culture with MOG, IL-1 β , and IL-23. (N) Cultured T cells were transferred to naive mice and clinical scores were recorded for EAE. (O) *Itga4* mRNA was quantified by RT-PCR in cultured cells before transfer. (P–R) CD4⁺ T cells were isolated from the spleens and LNs of mice immunized with MOG and CFA (day 7) and cultured for 3 d with IL-1 β , IL-23, and MOG, either in the presence or absence of V α 4⁺TCR β ⁺ cells. (P) IFN- γ in culture supernatants quantified by ELISA. (Q) *Ifng* expression in purified CD4⁺ T cells. (R) Proliferation of CD4⁺ T cells measured by CFSE dilution. Data are representative of three independent experiments ($n = 6$ for A–N) or combined from two experiments ($n = 6$ for O–R). Results are shown as mean \pm SEM; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$; one-way ANOVA with Tukey's test for multiple comparisons (A–C, F, and G), two-way ANOVA with Tukey's test for multiple comparisons (J), repeated measures (H, K, and N), or unpaired t test (I, L, and O–R).

Committee. Embryonic thymus experiments were performed with C57BL/6 mice at the University of Birmingham, UK. For timed matings, the day of detection of a vaginal plug was designated as day 0. Experiments with TCR α ^{-/-}, TCR β ^{-/-}, MHCII^{-/-}, and MHCII^{-/-} mice were performed at the Instituto de Medicina Molecular, Lisbon, Portugal.

Preparation of human PBMCs

PBMCs were isolated by Ficoll gradient centrifugation from leukocyte-enriched buffy coats obtained from anonymous healthy donors via the Irish Blood Transfusion Board, St. James's Hospital, Dublin, Ireland. Ethical approval was granted by the School of Biochemistry and Immunology Research Ethics Committee, Trinity College Dublin, Ireland.

Antibodies

The following antibodies were used to characterize murine cells in flow cytometry experiments: anti-CCR2 (clone SA203G11, 0.1 μ g/10⁶ cells; BioLegend), anti-CCR6 (clone 140706, 0.1 μ g/10⁶ cells; BD Horizon), anti-CCR7 (clone 4B12, 0.1 μ g/10⁶ cells; eBioscience), anti-CD3 (clone 17A2, 0.05 μ g/10⁶ cells; BioLegend), anti-CD4 (clone RM4-5, 0.05 μ g/10⁶ cells; eBioscience), anti-CD5 (clone 53-7.3, 0.1 μ g/10⁶ cells; BD Biosciences), anti-CD6 (clone IM348, 0.1 μ g/10⁶ cells; eBioscience), anti-CD8 α (clone 53-6.7, 0.05 μ g/10⁶ cells; eBioscience), anti-CD25 (clone PC61, 0.1 μ g/10⁶ cells; BioLegend), anti-CD27 (clone LG.7F9, 0.1 μ g/10⁶ cells; eBioscience), anti-CD49d (clone R1-2, 0.1 μ g/10⁶ cells; eBioscience), anti-CD86 (clone GL1, 0.1 μ g/10⁶ cells; BioLegend), anti-CD95 (clone 15A7, 0.1 μ g/10⁶ cells; eBioscience), anti-CD107a (clone 1D4B, 0.1 μ g/10⁶ cells; BD Biosciences), anti-CD115 (clone AFS98, 0.1 μ g/10⁶ cells; BioLegend), anti-CD122 (clone TM-b1, 0.1 μ g/10⁶ cells; eBioscience), anti-CD284 (clone MT5510, 0.1 μ g/10⁶ cells; BioLegend), anti-CX3CR1 (clone SA011F11, 0.1 μ g/10⁶ cells; BioLegend), anti-GITR (clone DTA1, 0.1 μ g/10⁶ cells; eBioscience), anti-GM-CSF (clone MP1-22E9, 0.1 μ g/10⁶ cells; eBioscience), anti-ICOS (clone 7E.17G9, 0.1 μ g/10⁶

cells; BioLegend), anti-IFN- γ (clone XMGL2, 0.1 μ g/10⁶ cells; eBioscience), anti-IL-1R1 (clone 12A6, 0.1 μ g/10⁶ cells; BD Biosciences), anti-IL-17 (clone TC11-18H10.1, 0.1 μ g/10⁶ cells; BioLegend), anti-IL-23R (clone 078-1208, 0.1 μ g/10⁶ cells; BD Biosciences), anti-TCR β (clone H57-597, 0.1 μ g/10⁶ cells; eBioscience), anti-TCR δ (clone GL3, 0.1 μ g/10⁶ cells; eBioscience), anti-TLR2 (clone T2.5, 0.1 μ g/10⁶ cells; BioLegend), anti-V α 2 (clone B20.1, 0.1 μ g/10⁶ cells; BioLegend), anti-V α 8.3 (clone B21.14, 0.1 μ g/10⁶ cells; BioLegend), anti-V γ 1 (clone 2.11, 0.1 μ g/10⁶ cells; BioLegend), and anti-V γ 4 (clone UC3-10A6, 0.1 μ g/10⁶ cells; BioLegend). The following antibodies were used to characterize human cells in flow cytometry experiments: anti-TCR $\alpha\beta$ (clone T10B9.1A-31, 5 μ l/test; BD Horizon), anti-CD3 (clone SK7, 1 μ l/test; BD Biosciences), and anti-V δ 2 (clone B6, 2.5 μ l/test; BioLegend).

Immune cell purification

Purified cell populations were isolated from single-cell suspensions of leukocytes extracted from spleens and LNs. Briefly, WT cells were enriched by magnetic separation using a Pan T Cell Isolation Kit (Miltenyi Biotec), labeled with anti-V γ 4 (clone UC3-10A6, 0.1 μ g/10⁶ cells; BioLegend) or anti-TCR δ (clone GL3, 0.1 μ g/10⁶ cells; eBioscience), sorted by flow cytometry, and incubated with anti-TCR β (clone H57-597, 0.1 μ g/10⁶ cells; eBioscience). Alternatively, WT cells were enriched by magnetic separation using a $\gamma\delta$ T Cell Isolation Kit (Miltenyi Biotec). In the EAE model, V α 4⁺TCR β ⁺ cells were enriched from spleens and draining LNs on day 10 after immunization with MOG and CFA. Distinct cell populations were sorted by flow cytometry using a FACSaria Fusion (BD Biosciences) or a MoFlo Legacy (Beckman Coulter).

Cell culture

Cells were cultured in medium with or without various combinations of IL-1 β , IL-4, IL-12p70, IL-18, and IL-23 (all 10 ng/ml), together with IL-2 (1 or 10 ng/ml) or anti-IFN- γ and anti-IL-17

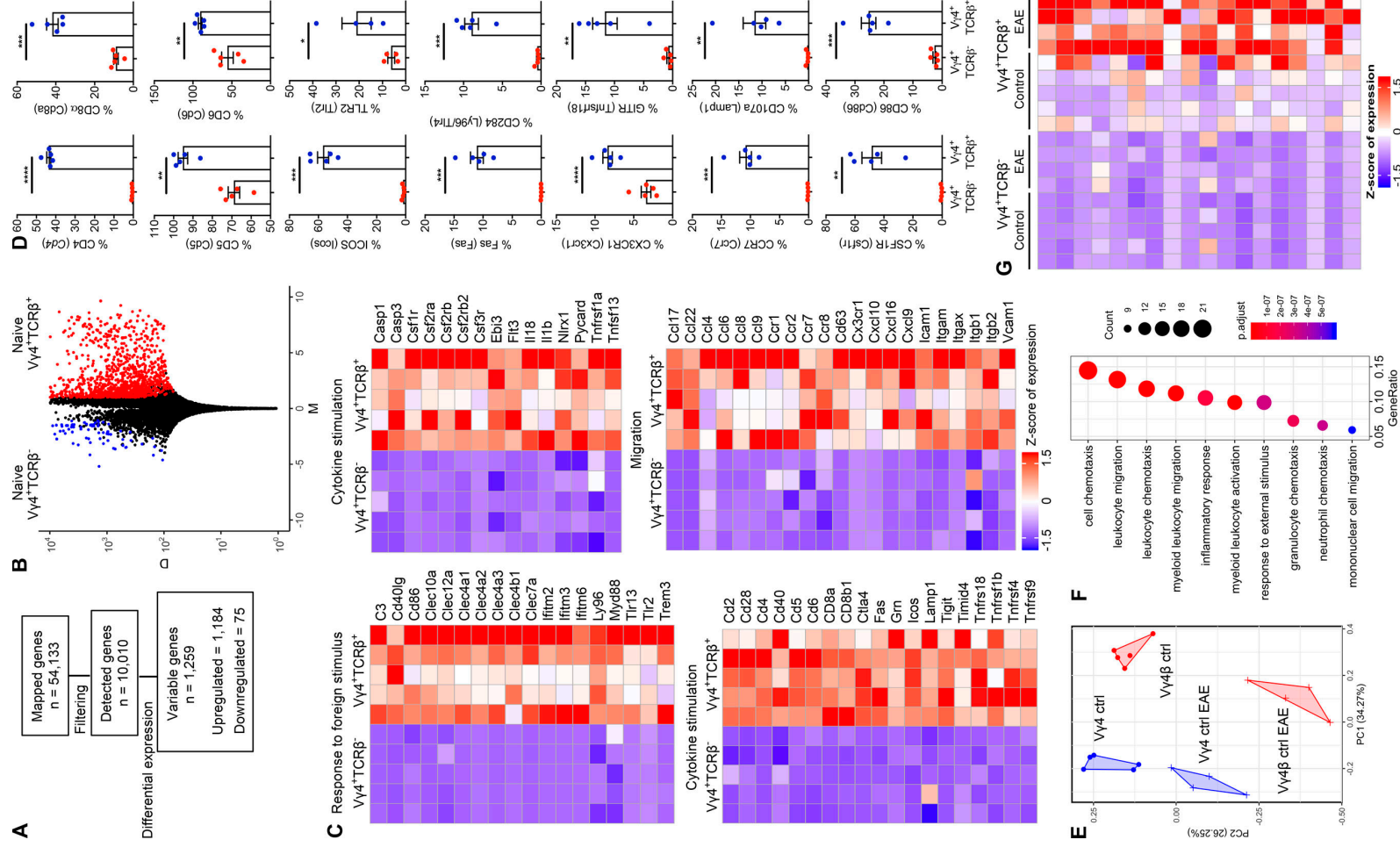


Figure 5. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells are transcriptionally distinct from conventional $\gamma\delta$ T cells. RNA sequencing analysis of $V\gamma 4^+TCR\delta^+TCR\beta^-$ ($V\gamma 4^+TCR\beta^+$) or $V\gamma 4^+TCR\delta^-TCR\beta^-$ ($V\gamma 4^+TCR\beta^-$) cells flow sorted from the spleens and LNs of naive WT mice or WT mice on day 3 of EAE. **(A)** Summary of the data pre-processing and filtering workflow. **(B)** NOISeq MD plot of genes passing the low-level filtering cutoff ($n = 10,010$), highlighting DEGs ($n = 1,259$) between naive $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells. Red dots: genes up-regulated in $V\gamma 4^+TCR\beta^+$ cells ($n = 1,184$). Blue dots: genes down-regulated in $V\gamma 4^+TCR\beta^+$ cells ($n = 75$). M represents the \log_2 -fold change in normalized expression values between $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells. D represents the absolute value of the difference in expression levels between $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells. D values are displayed on a \log_{10} scale. Increasing D values represent increasing differences in expression levels between $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells. **(C)** Heatmaps of selected genes from enriched biological processes derived using gene ontology enrichment analysis of up-regulated genes between naive $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells. Expression values were z-transformed for visualization. **(D)** Flow cytometric analysis of purified $CD3^+$ cells, comparing naive $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ cells, gated on live $CD3^+TCR\delta^+$ cells. Results are shown as mean \pm SEM. **(E)** Reduced dimensionality representation of four cell populations via a principal-component analysis plot, where the $V\gamma 4^+TCR\beta^+$ and $V\gamma 4^+TCR\beta^-$ populations separate along the first principal component (PC1) and the equivalent populations in naive mice or mice with EAE separate along the second principal component (PC2). **(F)** Dot plot of the top 10 significantly enriched biological processes inferred from differentially up-regulated genes in $V\gamma 4^+TCR\beta^+$ or $V\gamma 4^+TCR\beta^-$ cells from mice with EAE versus naive mice (cluster 1 in Fig. S3 C; $n = 158$ genes). Dot color represents the P-adjusted enrichment value, and dot size represents the number of genes within each enriched gene ontology. **(G)** Heatmap of genes associated with chemotaxis/migration among all four populations derived using the gene ontology enrichment analysis in F. Expression values were z-transformed for visualization. Most of the data are shown for individual mice ($n = 4$ or 5 mice per group), except in D, where the data are representative of two experiments ($n = 5$ mice). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$; unpaired t test.

(both 1 $\mu\text{g/ml}$), in the presence or absence of anti-CD3 (clone 145-2C11, 1 $\mu\text{g/ml}$; BD Biosciences), anti-CD28 (clone 37.51, 2 $\mu\text{g/ml}$; BD Biosciences), anti-MHCII (clone IA/IE, 10 $\mu\text{g/ml}$; eBioscience), and/or anti-TCR δ (clone UC7-13D5, 10 $\mu\text{g/ml}$; BioLegend). Culture supernatants were harvested 24–72 h after T cell activation, and concentrations of IFN- γ , IL-4, IL-17, and GM-CSF were quantified by ELISA (BD PharMingen or R&D Systems). For DC co-culture experiments, bone marrow from C57BL/6 mice was incubated with GM-CSF (20 ng/ml) for 6 d to generate mature DCs. Mature DCs were washed and incubated for 5 h at 20,000 cells/well (for culture with magnetically enriched $\gamma\delta$ T cells) or 2,500 cells/well (for culture with flow-purified populations) with medium alone (negative control), OVA_{257–264} (4 $\mu\text{g/ml}$; Sigma-Aldrich), OVA_{323–339} (10 $\mu\text{g/ml}$; Sigma-Aldrich), KLH (10 $\mu\text{g/ml}$; Calbiochem), or MOG_{35–55} (10 $\mu\text{g/ml}$; GenScript). Magnetically enriched $\gamma\delta$ T cells (10^5 cells/well from OT-I mice or 2×10^5 cells/well from OT-II mice), flow-purified $TCR\delta^+TCR\beta^+$ or $TCR\delta^+TCR\beta^-$ cells (20,000 cells/well) from OT-II mice, or flow-purified $V\gamma 4^+TCR\beta^+$ cells (20,000 cells/well) from mice immunized with MOG and CFA were then co-cultured in the presence or absence of various stimulants with mature DCs. Culture supernatants were harvested after 2 d and assayed by ELISA. Cells were harvested simultaneously into TRIzol Reagent (Thermo Fisher Scientific). For NKG2D ligand-induced activation, murine YAC-1 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS. WT cells were enriched by magnetic separation using a Pan T Cell Isolation Kit (Miltenyi Biotec) and incubated with or without YAC-1 cells at a 10:1 ratio for 48 h.

Ontogeny experiments

Thymuses from C57BL/6 embryos were isolated on E14, E16, and E18. Flow cytometric analyses were performed using either a FACSCantoII or an LSRFortessa (both BD Biosciences).

Flow cytometry

Flow cytometry was performed on LN cells, purified immune cell populations, and mononuclear cells enriched by Percoll density centrifugation from the brains and spinal cords of mice immunized with MOG and CFA. In all experiments, cells were

washed, stained with LIVE/DEAD Fixable Aqua (Life Technologies), and blocked with anti-CD16/CD32 (1 $\mu\text{g/ml}$; BD PharMingen). For intracellular staining, mononuclear cells were incubated for 5 h with brefeldin A (5 $\mu\text{g/ml}$; Sigma-Aldrich) in the presence or absence of phorbol myristate acetate (10 ng/ml; Sigma-Aldrich) and ionomycin (1 $\mu\text{g/ml}$; Sigma-Aldrich). Intracellular staining for IFN- γ , IL-17, and GM-CSF was conducted after fixation and permeabilization using a FIX&PERM Cell Permeabilization Kit (Caltag). Data were acquired using either a FACSCantoII or an LSRFortessa (both BD Biosciences) and analyzed with FACSDiva (BD Biosciences) or Flowjo software (Tree Star). Gates were set on isotype or fluorescence minus one controls. The gating strategy is depicted in Fig. S1 A.

Imaging flow cytometry

T cells were enriched from LNs by magnetic separation using a Pan T Cell Isolation Kit (Miltenyi Biotec) and labeled with anti-TCR β (clone H57-597, 0.1 $\mu\text{g}/10^6$ cells; eBioscience), anti-TCR δ (clone GL3, 0.1 $\mu\text{g}/10^6$ cells; eBioscience), and anti- $V\gamma 4$ (clone UC3-10A6, 0.1 $\mu\text{g}/10^6$ cells; BioLegend). Cells were then fixed and permeabilized, stained with DAPI (30 mM), and analyzed using an ImageStreamX Mark II Imaging Flow Cytometer (Amnis).

Library preparation and RNA sequencing

T cells were enriched from LNs by magnetic separation using a Pan T Cell Isolation Kit (Miltenyi Biotec), labeled as described above, and flow purified as $V\gamma 4^+TCR\delta^+TCR\beta^+$ or $V\gamma 4^+TCR\delta^+TCR\beta^-$ populations. Total RNA was isolated using a NucleoSpin RNA XS Kit according to the manufacturer's instructions (Clontech), omitting the carrier RNA and lysate filtration steps, and eluted in 10 μl of RNase-free water (Qiagen). RNA sequencing was performed on total RNA by normalizing the input mass to cell number according to cell type. For $V\gamma 4^+TCR\delta^+TCR\beta^-$ cells, input was normalized to 30,000 cells where possible (two samples fell below this input target, reaching totals of 12,687 and 21,772 cells). For $V\gamma 4^+TCR\delta^+TCR\beta^+$ cells, input was normalized to 5,000 cells where possible (three samples fell below this input target, reaching totals of 2,036, 3,649, and 4,577 cells). cDNA was amplified using a SMARTer Ultra Low Input RNA Kit for

Illumina Sequencing (Clontech) and sequenced at a target depth of 30×10^6 single-end (1×50 bp) reads per sample using a HiSeq 3000 System (Illumina).

Bioinformatic analysis of RNA sequencing data

Reads were mapped against the mouse reference genome (mm10, Ensembl release 76) using Kallisto (version 0.44; Bray et al., 2016). Transcript abundances were summarized to gene-level estimates in R (version 3.4.4) using the tximport Bioconductor package (version 1.8; Soneson et al., 2015) and the makeTxDbFromGFF package with a GTF annotation file from Ensembl (release 93). Differential expression analysis was performed using the NOISeq R Bioconductor package (version 2.22.1; Tarazona et al., 2015). Lowly expressed genes were removed on the basis of normalized read counts. Genes were kept if they reached a threshold of ≥ 100 read counts under any experimental condition. Data were normalized using the trimmed mean of log expression values approach in NOISeq. DEGs were detected using a probability value of 0.99, equivalent to a false discovery rate of 0.01, and an absolute fold-change cutoff of 2. A higher threshold of threefold was used for samples from mice with EAE, which exhibited higher levels of background noise. Heatmaps and clustering analyses, based on Euclidean distance with scaling by row, were generated using the ComplexHeatmap package in R (version 2.1.1; Gu, 2016). Principal-component analysis was performed using the R functions autoplot (ggfortify v0.4.8) and prcomp (stats v3.5.2) for all genes where the sum of the normalized read counts from all samples was at least 1,000 ($n = 10,010$). Gene ontology enrichment analysis was performed using the clusterProfiler package and the enrichGO tool in R (version 3.6.0; Yu et al., 2012).

Gene expression analysis

Total RNA was extracted from purified cell populations using the chloroform/isopropanol method. mRNA expression was evaluated by real-time PCR after reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed for a variety of genes using predesigned TaqMan Gene Expression Assays (Applied Biosystems). 18s rRNA was used as an endogenous control. Samples were assayed using a 7500 Fast Real-Time PCR System (Applied Biosystems).

TCR sequencing

For population-level analyses, viable $V\gamma 4^+ \text{TCR}\beta^+$ cells from WT mice were flow purified directly into RNeasy lysis buffer (Applied Biosystems). Unbiased amplification of all expressed *Tra*, *Trb*, *Trg*, and *Trd* gene rearrangements was conducted using a template-switch anchored RT-PCR (Quigley et al., 2011). Amplicons were subcloned, sampled, sequenced, and analyzed as described previously (Price et al., 2005). For single-cell analyses, viable $V\gamma 4^+ \text{TCR}\beta^+$ cells from WT mice were index-sorted into a 96-well plate, and expressed *Tra*, *Trb*, *Trg*, and *Trd* gene rearrangements were amplified using a previously described protocol with minor modifications (Dash et al., 2011). Briefly, direct lysis and reverse transcription were performed using SuperScript Vilo (Invitrogen). The resultant cDNA was subjected to a first-round PCR incorporating a cocktail of validated *Tra* and *Trb* primers

(Dash et al., 2011), together with newly designed *Trg* and *Trd* primers. The first-round products were subjected to a nested PCR using internal primer pools on separate 96-well plates for the α , β , γ , and δ chains, with each product assigned to an identical location for tracing back to the original cell. The final products were purified using Exonuclease I/Shrimp Alkaline Phosphatase. Sequencing was performed with the relevant constant region primers using an ABI Big Dye Sequencer (Applied Biosystems). Data were analyzed using a custom-built macro-enabled Microsoft Excel sheet to derive CDR3 nucleotide and amino acid sequences, and gene use was assigned by matching sequences against the IMGT database (Lefranc et al., 2009). If multiple species were observed, potentially reflecting biallelic rearrangements, the relevant transcripts were further resolved using additional PCRs. Briefly, the first-round products from the identified cell were subjected to another nested PCR using family-specific forward and reverse primers determined using a trace viewer. Resolution was further verified by adopting a “leave-one-out” amplification strategy, where the internal forward primer specific for the family determined in the previous experiment was withheld from the cocktail, and the resulting product was sequenced.

Immunofluorescence microscopy

$\gamma\delta$ T cells or $V\gamma 4^+$ cells were flow purified using a FACSARIA Fusion (BD Biosciences). Purified cells were transferred onto poly-L-lysine-coated chamber slides, incubated for 2 h at 37°C , fixed in 4% paraformaldehyde for 15 min, and blocked with 20% FCS for 20 min. $\gamma\delta$ T cells were immunostained with anti-TCR β -Alexa Fluor 647 and phalloidin, and $V\gamma 4^+$ cells were immunostained in 5% bovine serum albumin with rabbit anti-TCR β , washed, and labeled with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 594. Slides were mounted using Mounting Medium with DAPI (DakoCytomation) and viewed on a point-scanning confocal microscope (FV1000; Olympus). Confocal images were selected to represent at least 20 captures ($n = 3$ independent experiments).

S. aureus infection model

Mice were inoculated i.p. with *S. aureus* (5×10^8 CFU in 100 μl) and sacrificed after 3 h or 3 d. Peritoneal exudate cells (PECs) were isolated from infected mice by lavage of the peritoneal cavity with 3 ml of sterile PBS. Lavage fluid was centrifuged and assayed for IL-1 β , IL-17, and IFN- γ by ELISA. Kidneys were homogenized in 1 ml of sterile PBS. Total tissue bacterial load was established by plating serial dilutions of peritoneal lavage fluid or kidney homogenate on tryptic soy agar plates for 24 h at 37°C . Results were standardized to CFUs per milliliter.

EAE

Active EAE was induced by injecting mice s.c. with 100 μg of MOG_{35–55} peptide (GenScript) emulsified in CFA containing 4 mg/ml (0.4 mg/mouse) of heat-killed *Mycobacterium tuberculosis* (Chondrex). Mice were further injected i.p. with 250 ng of pertussis toxin (Kaketsuken) on days 0 and 2. In some experiments, mice were treated with anti- $V\gamma 4$ or an isotype control (250 μg /mouse; BioXCell), administered i.p. on days -1, 2, 5, 7, 11, 14, 17, and 20 of EAE. Passive EAE was induced by adoptive

transfer of MOG-specific cells. C57BL/6 mice were immunized s.c. with 100 μ g of MOG_{35–55} peptide (GenScript) emulsified in CFA containing 4 mg/ml (0.4 mg/mouse) of heat-killed *M. tuberculosis* (Chondrex). On day 10 after induction, the spleens and brachial, axillary, and inguinal LNs were removed from sacrificed mice and prepared as single-cell suspensions. For V γ 4 β -depleted cultures, cells were labeled with anti-TCR β (clone H57-597, 0.1 μ g/10⁶ cells; eBioscience) and anti-V γ 4 (clone UC3-10A6, 0.1 μ g/10⁶ cells; BioLegend), and V γ 4⁺TCR β ⁺ cells were depleted by flow cytometry. Cells were stimulated with combinations of IL-1 β (10 ng/ml), IL-23 (10 ng/ml), and/or MOG (100 μ g/ml) in complete medium at 10 \times 10⁶ cells/ml. After 72 h, cells were washed, and cytokine production in the supernatants was measured by ELISA. A total dose of 5 \times 10⁶ viable cells was injected i.p. into each naive C57BL/6 recipient. Mice were monitored daily for signs of clinical disease. Clinical signs of EAE were assessed according to the following scores: no symptoms, 0; limp tail, 1; ataxic gait, 2; hindlimb weakness, 3; hindlimb paralysis, 4; tetraparalysis/moribund, 5.

MOG tetramer staining

Draining LNs were removed from mice 7 d after immunization with MOG and CFA and cultured at 20 \times 10⁶ cells/ml in the presence of IL-2 (5 ng/ml), 2.5% FCS, and MOG or control tetramer (National Institutes of Health Tetramer Facility) for 2.5 h at 37°C. Naive mice were processed similarly as controls. Cells were then washed twice and analyzed by flow cytometry as described above. Gates were set on fluorescence minus one controls among live CD3⁺CD4⁺CD44⁺ cells.

Data availability

RNA sequencing data for purified V γ 4⁺TCR δ ⁺TCR β ⁺ or V γ 4⁺TCR δ ⁺TCR β [−] from LNs of naive mice or mice with EAE have been deposited to the Gene Expression Omnibus under accession no. GSE143500.

Statistical analysis

Statistical analyses were performed using one-way, two-way, and three-way ANOVAs and unpaired *t* tests in Prism (GraphPad). Error bars represent SD or SEM. Levels of significance are denoted as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; and ****, *P* < 0.0001.

Online supplemental material

Fig. S1 (related to Fig. 1) shows image and flow cytometry data from T cells that coexpress $\alpha\beta$ and $\gamma\delta$ TCRs. Fig. S2 (related to Fig. 4) shows activation data from hybrid $\alpha\beta$ - $\gamma\delta$ T cells. Fig. S3 (related to Fig. 5) shows RNA sequencing analysis of V γ 4⁺TCR β ⁺ and V γ 4⁺TCR β [−] cells before or after activation.

Acknowledgments

The authors thank Owen Hughes and Barry Lewis (Merck) for assistance with ImageStream analysis, Andreea Petrasca and Jean Fletcher for isolation of human PBMCs, and Seth Coffelt for technical assistance and discussions.

This work was supported by grants from Science Foundation Ireland (15/IA/3041 to R.M. McLoughlin and 11/PI/1036, 12/RI/

2340(7), 15/SPP/3212, and 16/IA/4468 to K.H.G. Mills), AbbVie (to K.H.G. Mills), Irish Higher Education Authority Program for Research in Third-Level Institutions (to S.C. Edwards and K.H.G. Mills), National Health and Medical Research Council (a CJ Martin ECR Fellowship to E.J. Grant), European Research Council (CoG_646701 to B. Silva-Santos), Medical Research Council (G1000213 to G. Anderson), Wellcome Trust (100326Z/12/Z to D.A. Price), National Institutes of Health (RO1AI107625 to P.G. Thomas), and American Lebanese Syrian Associated Charities (to P.G. Thomas).

Author contributions: S.C. Edwards and C.E. Sutton designed and performed most of the experiments; P. Dash and N. Apiwattanukul developed the single-cell method for sequencing murine $\gamma\delta$ TCRs; K. Ladell, E.J. Grant, J.E. McLaren, P. Dash, N. Apiwattanukul, and W. Awad sequenced TCRs; F. Roche and K. Hokamp analyzed the RNA sequencing data; K. Ladell, K.L. Miners, and B. Moran designed flow cytometry panels and sorted cells; J.C. Ribot performed the studies with TCR α ^{−/−}, TCR β ^{−/−}, MHCII^{−/−}, and MHCII^{−/−} mice; S. Baik performed the ontogeny studies; A. McGinley assisted with experimental work; S.J. Lalor and R.M. McLoughlin designed experiments and provided feedback on the manuscript; J. Paez-Cortez guided cell isolation and sorting methodologies for the single-cell RNA sequencing experiments; V. Pivorunas and L. Dowding designed and performed RNA extractions and designed the RNA sequencing input normalization strategy; M. Macoritto designed the sequencing strategy for single-cell RNA sequencing experiments; A. Slavin supervised and directed the RNA sequencing experiments with help from V. Pivorunas, L. Dowding, M. Macoritto, and J. Paez-Cortez; G. Anderson designed the ontogeny studies and provided feedback on the manuscript; B. Silva-Santos designed the studies with TCR α ^{−/−}, TCR β ^{−/−}, MHCII^{−/−}, and MHCII^{−/−} mice and provided feedback on the manuscript; D.A. Price and P.G. Thomas designed the TCR sequencing studies and provided feedback on the manuscript; K.H.G. Mills directed the project, designed the studies, and wrote the manuscript with help from S.C. Edwards, C.E. Sutton, and D.A. Price. All authors edited and approved the final manuscript.

Disclosures: Dr. Edwards reported grants from AbbVie during the conduct of the study. Dr. Thomas reported a patent to PCT/US2016/064735 pending. Dr. Mills reported grants from AbbVie during the conduct of the study and personal fees from Pieris outside the submitted work. In addition, Dr. Mills had a patent on IL-17 inhibitors pending. No other disclosures were reported.

Submitted: 9 May 2019

Revised: 29 November 2019

Accepted: 17 January 2020

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Supplemental material

Edwards et al.
T cells coexpressing $\alpha\beta$ and $\gamma\delta$ T cell receptors

Journal of Experimental Medicine
<https://doi.org/10.1084/jem.20190834>

S1

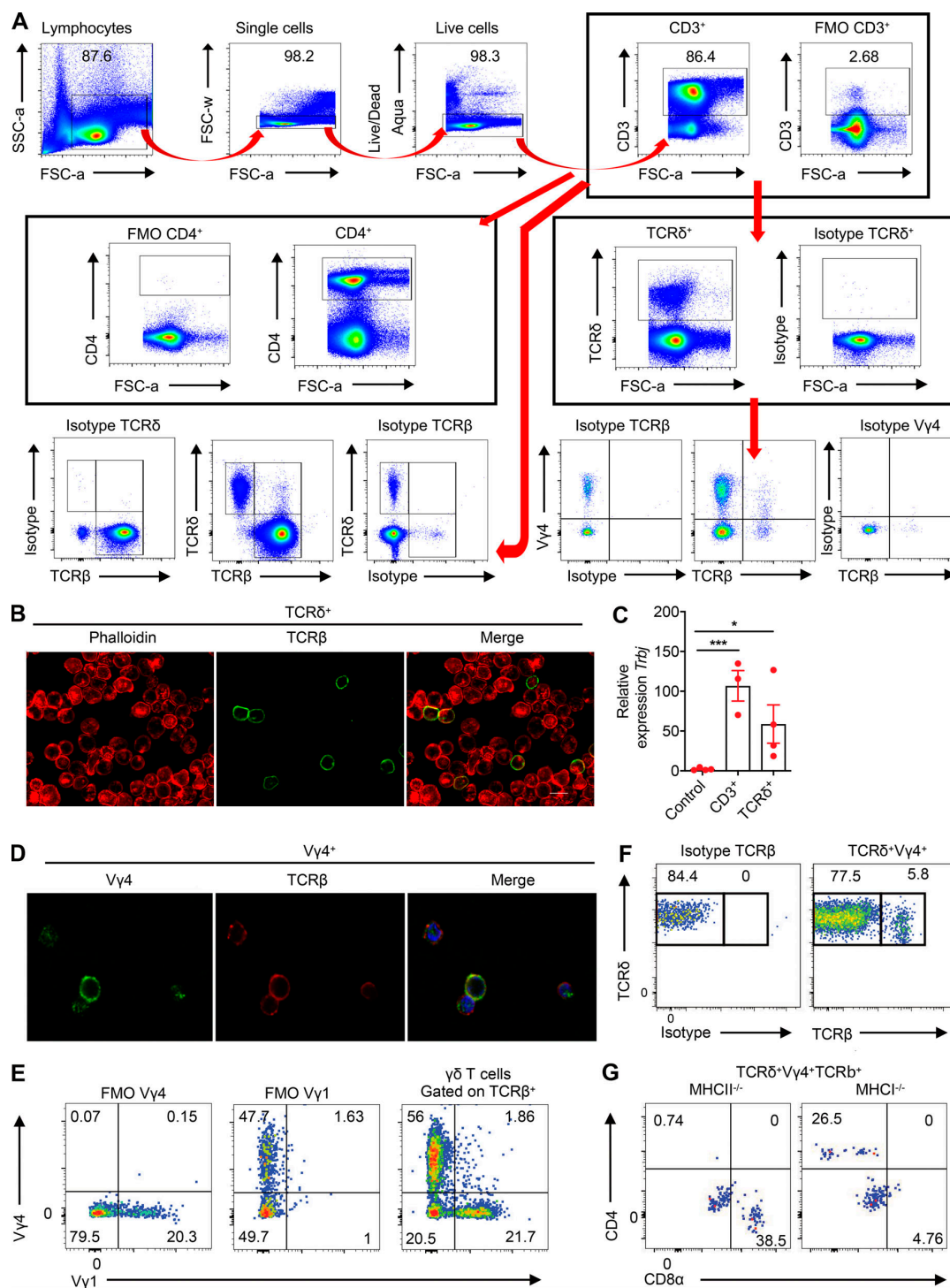


Figure S1. **A novel population of T cells that coexpresses $\alpha\beta$ and $\gamma\delta$ TCRs.** (A) Gating strategy for the analysis of T cell subsets, including hybrid $\alpha\beta$ - $\gamma\delta$ T cells, conventional $\gamma\delta$ T cells, and $CD4^+$ T cells. (B) Confocal images of purified TCR δ ⁺ cells costained for TCR β . (C) *Trb* expression in purified CD3⁺ or TCR δ ⁺ cells quantified by RT-PCR. Control: heart cells. *, $P < 0.05$, ***, $P < 0.001$. (D) Confocal images of purified CD3⁺V γ 4⁺ cells costained for TCR β . (E) Flow cytometry plots showing expression of V γ 1 versus V γ 4 on TCR δ ⁺ cells, gated on live TCR β ⁺ cells. (F) Flow cytometry plots showing expression of TCR δ versus TCR β on LN cells, gated on live CD3⁺V γ 4⁺TCR δ ⁺ cells. (G) Flow cytometry plots showing CD4 versus CD8 α on LN cells from MHCII^{-/-} or MHCII^{-/-} mice, gated on live TCR δ ⁺V γ 4⁺TCR β ⁺ cells. Data are representative of at least three independent experiments. Results are shown as mean \pm SEM. P values were calculated using a one-way ANOVA with Tukey's test for multiple comparisons (C). FSC, forward scatter.

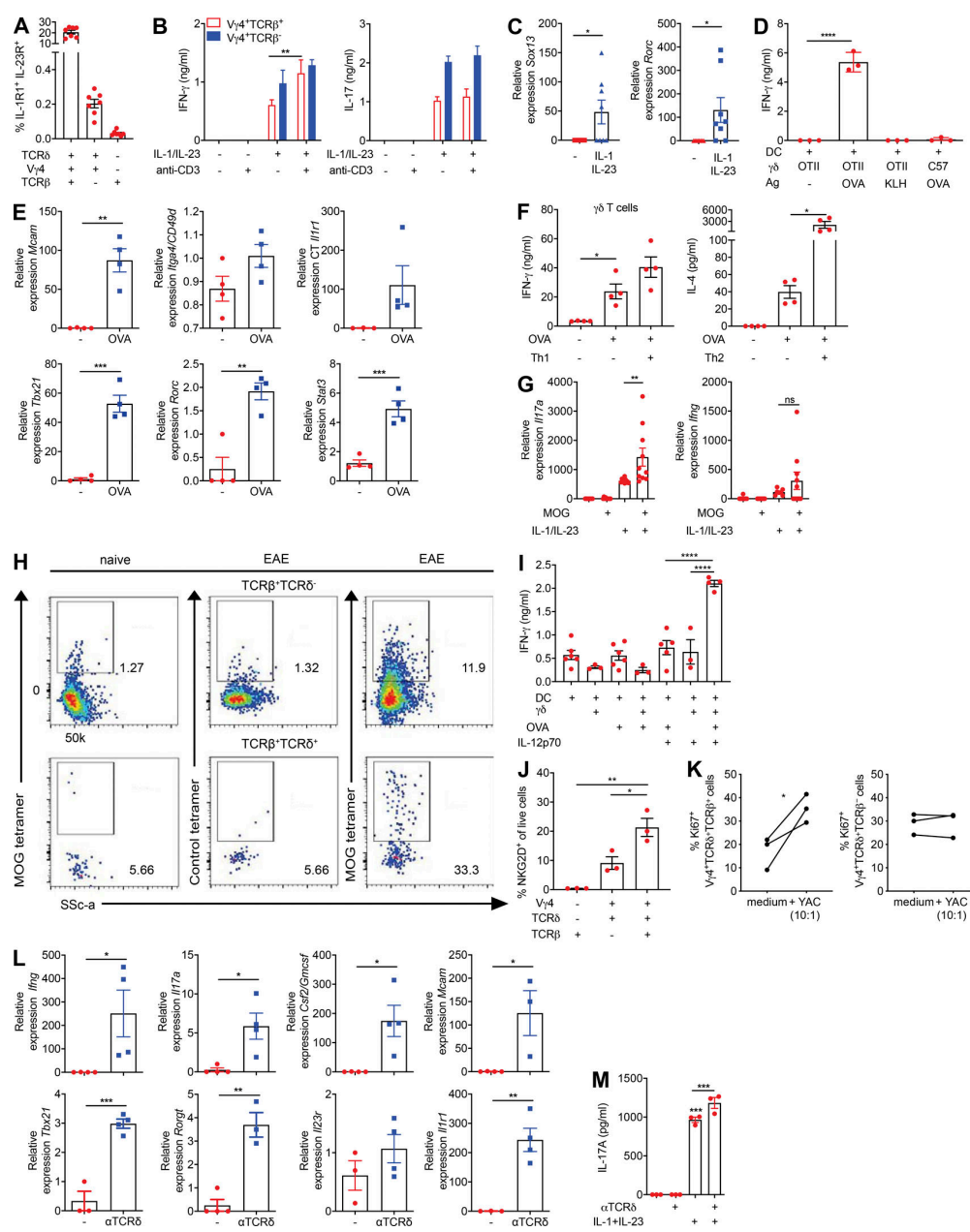


Figure S2. Hybrid αβ-γδ T cells can be activated innately or via αβ or γδ TCRs. (A) Coexpression of IL-1R1 and IL-23R on Vγ4⁺ hybrid αβ-γδ, Vγ4⁺ γδ T cells, or αβ T cells. (B) Production of IFN-γ and IL-17 by purified Vγ4⁺ TCRβ⁺ or Vγ4⁺ TCRβ⁻ cells (3,000 cells/well) stimulated for 3 d with IL-1β and IL-23 in the presence or absence of plate-bound anti-CD3. (C) Expression of *Sox13* and *Rorc* mRNA in purified Vγ4⁺ TCRβ⁺ cells stimulated for 2 d with IL-1β and IL-23. (D) Production of IFN-γ by TCRβ⁺ cells isolated from OT-II or C57BL/6 mice and cultured for 2 d with DCs pulsed for 5 h with OVA peptide or KLH. (E) Gene expression in TCRβ⁺ TCRδ⁺ cells isolated from OT-II mice and cultured for 3 d with DCs in the presence or absence of OVA peptide. (F) Cytokine production by γδ T cells isolated from OT-II mice stimulated for 3 d with or without OVA peptide in the presence or absence of IL-12p70 + IL-18 (Th1) or IL-4 + anti-IFN-γ + anti-IL-17 (Th2). (G) Gene expression in Vγ4⁺ TCRβ⁺ cells isolated from MOG-immunized mice on day 7 and cultured for 3 d with DCs in the presence or absence of MOG and/or IL-1β and IL-23. (H) LN cells isolated from 7 d MOG + CFA immunized or naive mice incubated with MOG-tetramer-Pe or control tetramer-Pe, gated on CD3⁺ CD4⁺ CD44⁺ cells, examining TCRβ⁺ TCRδ⁺ and TCRβ⁺ TCRδ⁻ populations. (I) γδ T cells from OT-I mice incubated for 3 d with DCs ± OVA peptide ± IL-12p70 and IFN-γ quantified in supernatants by ELISA. (J) Flow cytometry analysis of NKG2D expression on naive CD3⁺ T cells gating on TCRβ⁺ and TCRβ⁻ populations. (K) CD3⁺ T cells were incubated with and without YAC-1 cells (10:1) for 48 h. Proliferation was measured through expression of Ki67 by Vγ4⁺ TCRβ⁺ TCRδ⁺ versus Vγ4⁺ TCRβ⁺ TCRδ⁻ cells, gated on live CD3⁺ T cells. (L) Gene expression in TCRβ⁺ TCRδ⁺ cells stimulated for 2 d with anti-TCRδ. (M) Gene expression in CD3⁺ cells cultured for 3 d with IL-1β and IL-23 in the presence or absence of anti-TCRδ. Data are representative of at least two independent experiments. Results are shown as mean ± SEM. P values were calculated using a one-way ANOVA with Tukey's test for multiple comparisons (B, D, F, G, I-K, and M) or an unpaired t test (C, E, and L). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. ns, not significant.

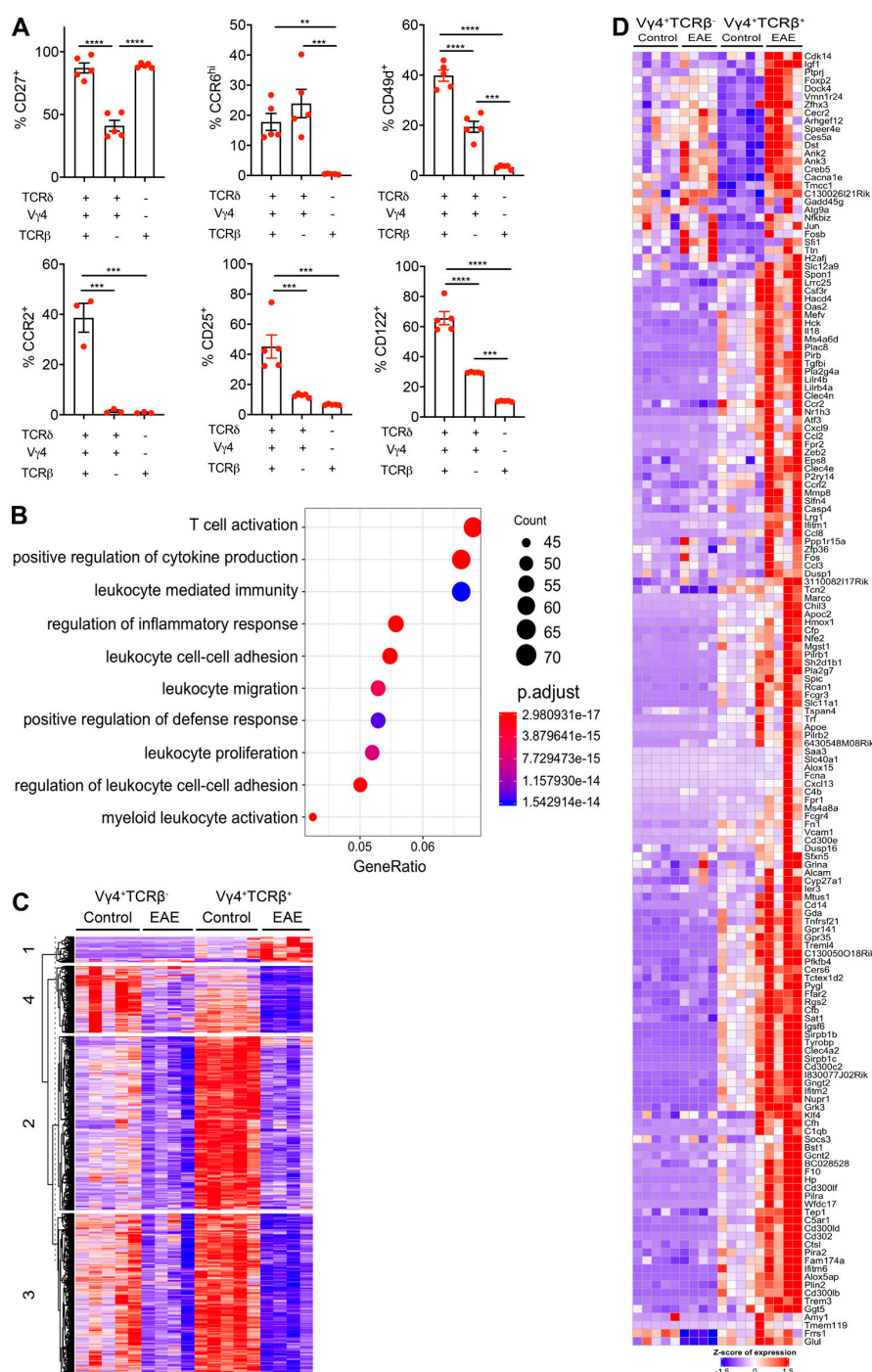


Figure S3. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells are transcriptomically distinct from conventional $\gamma\delta$ T cells and express Th17-associated markers. (A) Enriched T cells isolated from the spleens and LNs of WT mice were stained ex vivo for CCR2, CCR6, CD25, CD27, CD49d, and CD122. Expression was determined on live CD3⁺ cells coexpressing various combinations of V γ 4, TCR δ , and TCR β . Data are representative of at least two independent experiments. Results are shown as mean \pm SEM. P values were calculated using a one-way ANOVA with Tukey's test for multiple comparisons. (B) Dot plot of the top 15 significantly enriched biological processes inferred from differentially up-regulated genes in V γ 4⁺TCR β ⁺ versus V γ 4⁺TCR β ⁻ cells. Dot color represents the P-adjusted enrichment value, and dot size represents the number of genes within each enriched ontology. (C) Heatmap of all protein-coding genes that are differentially expressed in V γ 4⁺TCR β ⁺ or V γ 4⁺TCR β ⁻ cells from mice with EAE versus naive mice ($n = 2,686$). Genes are clustered using k-means clustering and a cluster size of 4. (D) Heatmap of all protein-coding genes that are up-regulated in either V γ 4⁺TCR β ⁺ or V γ 4⁺TCR β ⁻ cells from naive mice or mice with EAE (cluster 1 from Fig. S3 C, $n = 158$ genes). **, $P < 0.01$, ***, $P < 0.001$, and ****, $P < 0.0001$.